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Expression quantitative trait loci analysis of the Rubisco activase gene in maize

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Abstract

Expression quantitative trait loci (eQTL) analyses were applied in order to identify genetic factors that are relevant to the expression of a *β*-isoform Rubisco activase gene in maize, namely *ZmRCAβ*, in this study. During two years, a maize recombinant inbred line population was measured for *ZmRCAβ* expression levels at the grain filling stage. Based on a genetic map containing 916 molecular markers, we detected five eQTLs, namely *qRCA2.1* on chromosome 2, and *qRCA4.1*, *qRCA4.2*, *qRCA4.3*, and *qRCA4.4* on chromosome 4. These eQTLs explained the phenotypic variation ranging from 6.14% to 7.50% with the logarithm of the odd values ranging from 3.11 to 4.96. Based on the position of the eQTLs and *ZmRCAβ* on the chromosome, *qRCA4.2* was inferred as a *cis*-eQTL and the remaining as a *trans*-eQTL, suggesting that a combination of both *cis*- and *trans*-acting elements might control *ZmRCAβ* expression. *qRCA4.2, qRCA4.3*, and *qRCA4.4* were repeatedly detected during two years.

Additional key words: gene expression; grain yield; promoter; quantitative trait.

Introduction

Plants rely on Rubisco for carbon fixation (Lorimer 1981). Rubisco catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate under ample concentrations of CO_2 (Parry *et al.* 2003). In higher plants, the activity of Rubisco is regulated by another protein called Rubisco activase (RCA) (Portis 2003). RCA is a soluble chloroplast stromal protein that promotes the dissociation of RuBP or other inhibitory sugar-phosphates from an inactive Rubisco-sugar complex. Portis *et al*. (2008) described a model for the mechanism of RCA acting on Rubisco, where first RCA binds to Rubisco through electrostatic and other forces. Second, ATP hydrolysis promotes the movement of the C-terminal sensor-2 domain of RCA, and the N-terminal domain of Rubisco moves accordingly, which could break the interactions between Glu-60 in the N-terminal domain of Rubisco, Lys-334 in loop 6, and the bound sugar phosphate. Finally, loop 6 becomes free to move out of the active site, and the bound sugar phosphate is free to dissociate. In this way, RCA frees the active sites of Rubisco for spontaneous carboxylation by CO_2 and metal binding and activates the Rubisco holoenzyme.

Two forms of RCA (a *α*-isoform of 45–46 kDa and *β*-isoform of 41–43 kDa) are found in higher plants, and they differ only at the C-terminus (Salvucci *et al*. 1987, Portis 2003). Unlike the *β*-isoform, the *α*-isoform holds a C-terminal extension which contains the redox-sensitive Cys residues (Zhang and Portis 1999, Portis *et al*. 2008). The number of RCA-encoding genes varies depending on the plant species. Genomic analysis has identified one RCA gene in spinach, *Arabidopsis*, rice, and wheat (Werneke *et al*. 1988, To *et al*. 1999, Law and Crafts-Brandner 2001); two RCA genes in barley and cotton (Rundle and Zielinski 1991, Salvucci *et al*. 2003); and more than three RCA

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Abbreviations: eQTL – expression quantitative trait loci; gDNA – genomic DNA; LOD – logarithm of the odd; RCA – Rubisco activase; RIL – recombinant inbred line, RuBP – ribulose-1,5-bisphosphate.
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genes in tobacco (Qian and Rodermel 1993) and soybean (Yin *et al*. 2010). In some species, such as spinach, *Arabidopsis*, and rice, two RCA isoforms arise from alternative splicing of one RCA gene transcript (Werneke *et al*. 1989, Rundle and Zielinski 1991, To *et al*. 1999), whereas different genes encode the two RCA isoforms in other species, such as cotton and soybean (Salvucci *et al*. 2003, Yin *et al*. 2010).

Numerous studies have shown that endogenous levels of RCA are important for plant photosynthesis, making the modulation of RCA expression a promising target for improving crop yield. In eleven European cultivars of winter wheat, a significant positive, linear correlation was observed between the expression of RCA and plant productivity under heat-stress conditions (Ristic *et al*. 2009). In rice cultivars, overexpression of the RCA *α*-isoform improved the grain yield, while antisense-expression plants showed an opposite effect (Wu *et al*. 2007). In 184 soybean recombinant inbred lines (RILs), the expression of two RCA genes was positively correlated with Rubisco activity, photosynthetic rate, and seed yield (Yin *et al*. 2010). Similarly, a recent study showed that the expression level of the same two RCA genes was positively correlated with chlorophyll fluorescence parameters and seed yield in 219 soybean landraces (Chao *et al*. 2014). Thus, an analysis of the determinants of RCA expression not only helps to understand the genetic basis of RCA regulation, but also provides an approach to improve crop yield by altering RCA expression levels to optimize Rubisco activation under the prevailing environmental conditions (Chao *et al*. 2014).

Expression quantitative trait locus (eQTL) mapping has been widely used to identify the determinants of gene expression (Yin *et al*. 2011, Chao *et al*. 2014, Song *et al*. 2014). This approach treats gene expression as quantitative traits in a segregating population and maps eQTLs that control expression levels *in vivo* (Jansen and Nap 2001). eQTLs can be classified as *cis*- or *trans*-acting, depending on whether the eQTL is located close to the target gene (Doss *et al*. 2005). If the position of the target gene and its eQTL are congruent, the eQTL is called a *cis*-eQTL, suggesting that the allelic polymorphism of the gene itself, or a closely linked regulatory elements, directly impact the gene's expression; if not, the eQTL is termed *trans*-eQTL, indicating that gene expression is mainly regulated by *trans*-acting factors. In our previous studies using 184 soybean RILs and 219 soybean landraces, both *cis*- and *trans*-eQTLs were detected for soybean RCA genes (Yin *et al*. 2010, Chao *et al*. 2014).

Maize (*Zea mays*), a C_4 plant, is one of the most important crops in the world, serving as an essential source of food, feed, and fuel. Studies have shown that the expression level of RCA plays an important role in yield formation in this species. In genetically related population of the same maize cultivar, the high-yielding population had higher levels of RCA than the low-yielding population under field-grown conditions (Martínez-Barajas *et al*. 1997), and an improved the maize grain yield by a mass selection procedure was basically due to enrichment of leaf RCA content (Morales *et al*. 1999). In 123 maize inbred lines with extensive genetic diversity, the transcript abundance and protein expression levels of two RCA genes were positively correlated with the grain yield, and the correlation with the grain yield for the expression of the two genes was even larger than that for three C_4-specific photosynthesis high-efficiency genes (Yin *et al*. 2014b). However, to date, little information about the eQTL that regulates maize RCA gene expression is available.

Recently, we cloned two RCA genes from maize, the *α*- and *β*-isoform of RCA-encoding gene, namely *ZmRCAα* and *ZmRCAβ*, and observed that the correlation coefficient between *ZmRCAβ* and yield was higher than that between *ZmRCAα* and yield (Yin *et al*. 2014b). In the present study, we mapped eQTLs for *ZmRCAβ* in a RIL population derived from two maize inbred lines, RA and M53, in two growing seasons. In addition, as nucleotide changes in both coding and noncoding regions including promoters can significantly affect gene expression, we also examined the sequence variation in these regions of *ZmRCAβ* from RA and M53. Our results could be used for regulating RCA gene expression in maize breeding program.

Materials and methods

Plant material and growth conditions: A maize RIL population derived from a cross between maize inbred lines RA and M53 was used to determine the expression level of *ZmRCAβ* and maize grain yield. This population consists of 228 F8:10 lines derived *via* single-seed descent and has been used for mapping QTLs for resistance to *Aspergillus flavus* infection (Yin *et al*. 2014a) and chlorophyll *a* fluorescence parameters and grain yield (Yin *et al*. 2015). The parent inbred lines RA and M53 were used for sequencing the coding and noncoding regions including promoter genomic DNA (gDNA) of *ZmRCAβ*.

The RILs and parents were planted during two growing seasons at the experimental farm of the Agricultural College of Yangzhou University. Seeds were sown on the 20 June 2014 and on the 2 April 2015. In each growing season, all lines were planted with two replicates in a randomized complete-block design. Each line was planted in one row per plot; the length of each row was 2.5 m, the spacing between plants in each row was 0.25 m, and the spacing between rows was 0.55 m. The planting experiment was conducted under natural conditions. The cultivation management protocol followed local standard practices in each growing season.

Tissue preparation: For gene expression measurement, a previously described procedure for tissue preparation was

used (Yin *et al*. 2014b). Briefly, at 32 d after anthesis, the leaves closest to the ear were collected individually from three randomly selected plants of each RIL in the morning (09:00–12:00 h) on a sunny day, frozen immediately in liquid nitrogen, and stored at –70°C for *ZmRCAβ* expression measurement. In order to reduce the effects of maturity time on gene expression evaluation, we excluded twenty lines with extremely early or late maturity time based on their anthesis time. Thus, the RILs used for gene expression measurement were at a similar growth stage when collecting the leaf samples.

***ZmRCAβ* expression**: We used real-time quantitative PCR (RT-PCR) to measure *ZmRCAβ* expression level. Two samples for each genotype, each consisting of 15 mg of leaves from three plants in each plot, were used for measurement. The samples were ground with a mortar and pestle in liquid nitrogen until pulverized. Total RNA was isolated from leaves using an RNA plant extraction kit (*Vazyme*, China), and approximately 2 μg of purified total RNA was reverse transcribed using *HiScript* reverse transcriptase (*Vazyme*, China) with random primers. The RT-PCR assay was performed according to previously described procedures (Yin *et al*. 2010, 2014b). The constitutively expressed actin gene (GenBank accession no. J01238) was used as an endogenous reference. gene-specific primers (Table 2S, *supplement available online*) were used for real-time quantitative RT-PCR assay. Mixed cDNA from different inbred lines, a common experimental sample, was used as the calibrator on each RT-PCR plate. Normalized expression for each line was calculated as:

$$\Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,Actin})_{genotype} - (C_{T,\,Target} - C_{T,\,Actin})_{calibrator}.$$

Grain yield was estimated using the average yield of five plants in the middle of each row. At maturity, the ears of the corresponding plants were hand harvested, dried to a constant mass, and threshed, and the mean grain yield per plant was recorded.

***ZmRCAβ* genomic DNA sequence analysis**: Genomic DNA (gDNA) was extracted from the young leaves of maize plants using the CTAB method (Murray and Thompson 1980). Full-length gDNA of *ZmRCAβ*, including its promoter sequence, was amplified from RA and M53 using the G-promoter and G-RCAβ primers (Table 2S). PCR was conducted in a 50 μl reaction volume using *MAX* polymerase (*Vazyme*, China) following the manufacturer's recommendations, using a *C-1000* thermal cycler (*Bio-Rad*, USA). The cycling program consisted of one cycle at 95°C for 5 min, 35 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 2 min and one cycle at 72°C for 5 min. Amplification products were separated by electrophoresis on 1% agarose, and the band of an expected size was excised and sequenced at *BGI* (Shanghai, China). All sequences were checked manually, and sequence alignment was conducted using *DNAMAN* software (http://www.lynnon.com/).

Statistical analysis and eQTL/QTL mapping: *ZmRCAβ* abundance data and grain yield data for the RILs were analyzed using the *SAS* system (*9.0 for Windows*). An analysis of variance (*ANOVA*) was performed using *SAS PROC GLM*. The mean values of each trait for each RIL were calculated using *SAS PROC MEANS*. The *Pearson*'s phenotypic correlations between the traits were calculated using *SAS PROC CORR*.

eQTL/QTL analysis allows the genetic basis of variation of quantitative traits of interest to be dissected. Scoring every individual of a mapping population for the trait of interest and establishing a genetic linkage map for that population are two prerequisites for eQTL/QTL detection. In this study, the mean values of *ZmRCAβ* expression level and grain yield for RILs and a previously described linkage map (Yin *et al*. 2014a) were used for eQTL/QTL analysis. The linkage map of this RIL population covered 1,367 centimorgan of the maize genome and converted into ten linkage groups consisting of 916 molecular markers. The average distance between markers was 1.50 cm.

eQTL/QTL analysis was performed using a *QTL Cartographer version 2.5* (Wang *et al*. 2012). *Model 6* of composite interval mapping was deployed for mapping eQTLs/QTLs and estimating their effects. The genome was scanned at 2 cm intervals, and the forward regression method was selected. An empirical log of the likelihood odds (LOD) threshold of 2.5 was used for declaring an eQTL/QTL. The maximum LOD score along the interval was taken as the position of the eQTL/QTL, and the region in the LOD score within 1 LOD unit of the maximum was taken as the confidence interval. The additive effects of the detected eQTL/QTL were estimated as the mean effects of replacing both M53 alleles with RA alleles at the locus studied. Thus, for an eQTL/QTL to have a positive effect, the RA allele must increase the trait value. The contribution of each identified eQTL/QTL to the total phenotypic variance (R^2) was estimated using variance component analysis. eQTL/QTL nomenclature was adapted as described previously (Yin *et al*. 2010), thereby starting with a "q", followed by an abbreviation of the trait name, the name of the linkage group, and the number of eQTL/QTL affecting the trait on the linkage group.

Results

Quantitative variation in RIL families and characterization of the parental lines: There was considerable variability in *ZmRCAβ* expression level and grain yield among the RILs across different environments (Table 1). *ZmRCAβ* expression level was significantly affected by genotypes and genotype × environment interactions. There

Table 1. Descriptive statistics, *ANOVA*, and heritability estimates of *ZmRCAβ* expression levels and grain yield. ** – $P<0.01$; NS – not significant. G – genotype; E – environment; H^2 – broad-sense heritability.

Trait	Year	RA Mean ± SD	M53 Mean ± SD	RILs Mean ± SD	Range	Skew	G[a]	G × E[b]	H^{2c} [%]
ZmRCAβ expression	2014	2.62 ± 1.33	0.41 ± 0.08	1.37 ± 1.06	0.18–6.54	2.19	4.93**	2.31**	67
	2015	2.48 ± 0.64	0.31 ± 0.19	0.75 ± 0.68	0.02–7.27	3.71			64
Grain yield [g]	2014	22.83 ± 1.76	83.70 ± 7.32	33.97 ± 16.85	4.84–124.21	1.20	4.58**	0.95NS	57
	2015	17.36 ± 1.28	72.37 ± 3.54	28.20 ± 17.54	4.43–119.87	1.25			59

were markedly significant differences in the genotypes but no genotype × environment interactions for the grain yield. RA had a higher *ZmRCAβ* expression level than that of M53, but was lower than that of M53 in the grain yield.

Transgressive segregation in both directions was observed for *ZmRCAβ* expression level and grain yield. Heritability (H^2) was low to moderate for *ZmRCAβ* expression level and grain yield, ranging from 57 to 67%, which indicated that these two traits could be influenced, to a large extent, by the environment in which the plants were grown.

Correlation between *ZmRCAβ* expression and grain yield: In 2014 growing season, the *ZmRCAβ* expression level was significantly correlated with the grain yield. However, in 2015 growing season, no significant correlation was observed between *ZmRCAβ* expression level and the grain yield. *Pearson*'s phenotypic correlation coefficient between *ZmRCAβ* expression level and the grain yield was 0.214 in the 2014 growing season and 0.141 in 2015.

eQTL and QTL mapping: Five eQTLs for *ZmRCAβ* expression were detected on two chromosomes (Table 2, Fig. 1). eQTL *qRCA4.2*, *qRCA4.3*, and *qRCA4.4* on chromosome bins 4.02 and 4.03 explained 5–7% of the total phenotypic variation. Additive effect values indicated that RA alleles increased *ZmRCAβ* expression level at these three loci. Notably, all of these eQTLs were detected stable across two environments. The eQTLs *qRCA2.1* and *qRCA4.1* were detected only in one environment.

According to previous studies, a *cis*-eQTL was defined as being located within 5 Mb of the target gene; otherwise, it was termed a *trans*-eQTL (Morley *et al*. 2004, Chao *et al*. 2014). We compared the position of the five detected eQTLs with that of *ZmRCAβ* on maize chromosomes by surveying the reference genome sequence of the inbred line B73 (http://www.phytozome.net/maize). Among the five detected eQTLs, *qRCA4.2* was located on the same chromosome as *ZmRCAβ* and was 4.55 Mb away from *ZmRCAβ*, suggesting that this eQTL might be a *cis*-eQTL; the remaining eQTLs were located either on different chromosomes or on the same chromosome but with more than 5 Mb of physical distance away from *ZmRCAβ*, suggesting that these eQTLs might be *trans*-eQTLs.

Four QTLs for the grain yield were revealed, namely QTL *qSY1.1*, *qSY1.2*, *qSY8.1*, and *qSY9.1* in chromosome bins 1.05, 1.05, 8.00, and 9.06, respectively (Table 2, Fig. 1). All of these QTLs were detected in only one environment, in either the 2014 growing season or the 2015 growing season. *qSY1.1* and *qSY1.2,* respectively, explained 5.11% and 5.31% of the phenotypic variation with positive alleles from RA. *qSY8.1* and *qSY9.1,* respectively, explained 4.68% and 5.69% of the phenotypic variance with positive alleles from M53 (Table 2). The QTL *qSY9.1* had previously been detected for maize grain yield (Yin *et al*. 2015).

Sequence variation between RA and M53 in *ZmRCAβ* full-length genomic DNA and promoter region: Because nucleotide changes in both coding and noncoding regions including the promoter can significantly affect gene expression (Kumar *et al*. 2010), we were interested in the sequence variation among these regions between inbred lines RA and M53, the parents of the RIL population used in the present study. According to the annotation of the *ZmRCAβ* sequence in the maize inbred line B73 genome (http://www.ncbi.nlm.nih.gov/gene/?term=ZmRCA1), the length of the promoter, the 5′-UTR, exons, introns, and 3′-UTR of *ZmRCAβ* were 517, 137, 1302, 261, and 164 bp, respectively (Fig. 2*A*). Based on this observation, we amplified the corresponding gDNA region of *ZmRCAβ* from RA and M53 (Fig. 2*B*). Comparison of *ZmRCAβ* gDNA sequences from RA and M53 using *DNAMAN* software showed obvious differences between these two inbred lines, especially in the promoter region (Fig. 1S, *supplement available online*). Sequence alignment revealed 22 polymorphic sites (Fig. 2*B*). Three of six polymorphic sites in the promoter, including SNPs and indels, were located in the regulatory elements, such as the TATA-box, Sp1, TC-rich repeats, G-box, and AE-box (Table 1S, *supplement available online*). Interestingly, the 390-bp indel contained all of the regulatory elements mentioned above. There were eight polymorphic sites in the 3′-UTR, six polymorphic sites in the exons, and two polymorphic sites in the introns. The three-base indel in the exon2 resulted in a deletion of serine in RA, and the second SNP in the exon3 caused a change in amino acid from glycine in M53 to asparagic acid in RA (Fig. 2S, *supplement available online*). In contrast, no polymorphic sites in the 5′-UTR were observed (Fig. 2*B*).

Discussion

Transgressive lines were observed with higher and lower *ZmRCAβ* expression levels than the max/min of parental lines (Table 1). The complementary genetic structure between parents is a general explanation for transgressive segregation in the plant genetics literature (Rieseberg *et al*. 1999). Consistent with this phenomenon, an eQTL with a positive allele from either RA or M53 was detected (Table 2).

Five eQTLs were detected on chromosomes 2 and 4 in two growing seasons, explaining 6.14 to 7.50% of the total phenotypic variation (Table 2, Fig. 1). Notably, three eQTLs in chromosome bins 4.02 and 4.03 were repeatedly detected in two growing seasons, suggesting that major eQTLs, which regulate *ZmRCAβ* expression, are present in these chromosome bins. Recently, a genome-wide association analysis using 123 maize inbred lines and 117 SSR markers detected one eQTL controlling *ZmRCAβ* in chromosome bin 6.01 in one field growing season (Yin *et al*. 2014b). This eQTL was not detected in the present study. Possible reasons for no common detected eQTLs between these two studies may be low marker density, phenotyping error, statistical defects with genome-wide association analysis (Zhao *et al*. 2011), or differences in the mapping population (Druka *et al*. 2010). Similar results were also observed in our previous studies mapping eQTLs for soybean RCA genes. We mapped different eQTLs for soybean RCA genes when using a RIL population (Yin *et al*. 2010) compared to 219 soybean landraces (Chao *et al*. 2014).

In our previous study, *ZmRCAβ* expression was highly correlated with the grain yield and had one eQTL that coincided with QTL for grain yield in 123 maize inbred lines, strongly suggesting that *ZmRCAβ* plays an important role in determining maize productivity (Yin *et al*. 2014b). However, in the present study, in the two parents, RA and M53, a higher *ZmRCAβ* expression seemed to result in the lower grain yield (Table 1). In addition, the correlation coefficients between *ZmRCAβ* expression and grain yield were relatively small, and no coincident eQTL/QTLs were detected between these two traits (Fig. 1). A possible reason for these differences is that the maize RIL population and its parents used in the present study might contain surplus *ZmRCAβ* expression. Consistent with this hypothesis, over- and anti-sense expressions of RCA resulted in significant changes of plant steady-state photosynthesis and/or grain yield in some cases (Wu *et al*. 2007), whereas they caused no obvious changes, unless the RCA content was reduced to a threshold level, in other cases (von Caemmerer *et al*. 2005, Yamori *et al*. 2012). Grain yield is a complex trait that is regulated by a number of elementary factors, and not every elementary factor is equally effective in determining the grain yield (Zhang *et al.* 2015). Only the limiting factors would have a high correlation with the grain yield, and thus a coincident eQTL/QTL would be detected (Yin *et al.* 2010). Accordingly, we must conclude that genes other than *ZmRCAβ* limit the grain yield in the RIL population and its parents used in the present study.

In the present study, both *cis*- and *trans*- eQTLs were revealed for *ZmRCAβ* in a maize RIL population (Table 2, Fig. 1). Similarly, in a recent study, we reported that soybean *GmRCAβ* expression is also controlled by both *cis*- and *trans*- eQTLs (Chao *et al*. 2014). These findings suggest that RCA gene expression can be controlled by a combination of both *cis*- and *trans*-acting elements. Consistent with these findings, interactions of multiple *trans*-acting protein factors with multiple cognate *cis*-acting DNA elements affect RCA gene expression (Schindler *et al*. 1992). Recently, it was shown that rice RCA gene expression in response to light was affected not only by a promoter but also by some nuclear proteins (Yang *et al.* 2012). Identifying the elements responsible for the eQTLs detected in the present study would help further the understanding of the transcriptional regulation mechanism of *ZmRCAβ* gene expression.

Sequence variation in coding regions, noncoding regions, and the promoters of target gene are usually regarded as elements responsible for *cis*-eQTL (Jansen and Nap 2001, Druka *et al.* 2010, Kumar *et al.* 2010, Chao *et al.* 2014). In the present study, extensive sequence variations in introns, exons, the 3′-UTR, and promoter of *ZmRCAβ* were observed between RA and M53 (Fig. 2*B*). These variations might be factors that generate diversity at the *ZmRCAβ* expression level in the RILs derived from RA and M53 and thus can be regarded as candidate *cis*-elements responsible for the *cis*-eQTL *qRCA4.2* which was detected in the present study. Notably, a 390-bp indel in the promoter of *ZmRCAβ* was located in the regulatory elements, and above all, the 390-bp indel contained five regulatory elements, including a TATA-box, Sp1, TC-rich repeats, a G-box, and an AE-box (Table 1S). This indel might be the most important factor in the promoter for the variation of *ZmRCAβ* expression. In addition to the promoter, downstream-like elements at the 3′-UTR were suggested to be involved in the circadian expression of the maize RCA transcripts (Ayala-Ochoa *et al.* 2004). Direct evidence has shown that changes in the 3′-UTR stabilized RCA transcript levels during heat stress in *Arabidopsis* (DeRidder *et al*. 2012), and promoter sequence types influenced RCA gene expression in soybean (Chao *et al*. 2014). Further studies should be conducted to characterize the important *cis*-elements that regulate *ZmRCAβ* expression. A thorough understanding of the function of the *cis*-elements regulating *ZmRCAβ* expression can be helpful for its exploitation in biotechnological applications.

Modulation of RCA expression is an attractive experimental goal for the improvement of crop performance (Wu *et al*. 2007, Yin *et al*. 2010, Yamori *et al*. 2012, Chao *et al.* 2014). Mapping eQTL for RCA genes should have a direct application in maize breeding

Table 2. Principal characteristics of eQTLs/QTLs for *ZmRCAβ* expression levels and grain yield. eQTL/QTL is named according to Yin *et al.* (2010): starting with "q", followed by an abbreviation of the trait name, the name of the linkage group, and the number of the eQTL/QTL affecting the trait on the linkage group. RCA – *ZmRCAβ* expression level; SY – grain yield. Marker intervals within which eQTL/QTL was mapped. Position – position from the first marker on each linkage group. LOD – maximum LOD score at the eQTL/QTL position. LOD is the probability associated with the most likely location of the detected eQTL/QTL. R^2 – coefficient of determination or the percentage of the phenotypic variance that is explained by the detected eQTL/QTL. Add – estimated phenotypic effect of substituting both M53 alleles with RA alleles at eQTL/QTL. Confidence intervals were set as the map interval corresponding to a 1-LOD decline on either side of the LOD peak.

Trait	eQTL/QTL	Bin	Marker interval	Position [cm]	2014				2015				Combined			
					LOD	R^2 [%]	Add	Confidence interval	LOD	R^2 [%]	Add	Confidence interval	LOD	R^2 [%]	Add	Confidence interval
ZmRCAβ expression	qRCA2.1	2.07	PZE-102147840–PZE-102154251	85.06	4.96	7.50	0.30	84.7–85.9					4.36	6.37	0.18	84.5–86.3
	qRCA4.1	4.00	umc1232-PZE–104157101	0.01	3.93	6.14	–0.28	0.0–4.0					3.75	5.60	–0.18	0.0–5.1
	qRCA4.2	4.02	PZE-104007604–SYN7819	36.46	4.01	6.27	0.31	35.3–37.2	4.55	6.83	0.19	35.9–37.1	5.41	8.39	0.24	35.6–37.1
	qRCA4.3	4.02	SYN5712–umc1371	40.34	3.54	6.44	0.31	39.6–40.6	3.87	6.61	0.18	39.6– 0.6	3.37	5.90	0.20	39.4–40.7
	qRCA4.4	4.03	PZE-104016174–PZE-104150421	46.38	3.11	5.31	0.28	46.0–46.9	3.43	5.82	0.16	46.0– 6.8				
Grain yield	*qSY1.1*	1.05	PZE-101100634–PZE-101107894	99.48					2.85	5.11	4.28	99.1–100.1				
	qSY1.2	1.05	PZE-101117507–PZE-101107138	100.70					2.96	5.31	4.33	100.1–101.9				
	qSY8.1	8.00	PZA01209.1–PZE-108000665	2.01	2.68	4.68	–3.97	1.2–5.6								
	qSY9.1	9.06	PZE-109099670–PZE-109104633	121.03	3.23	5.69	–5.89	117.0–122.5					2.81	4.68	–4.87	120.7–122.3

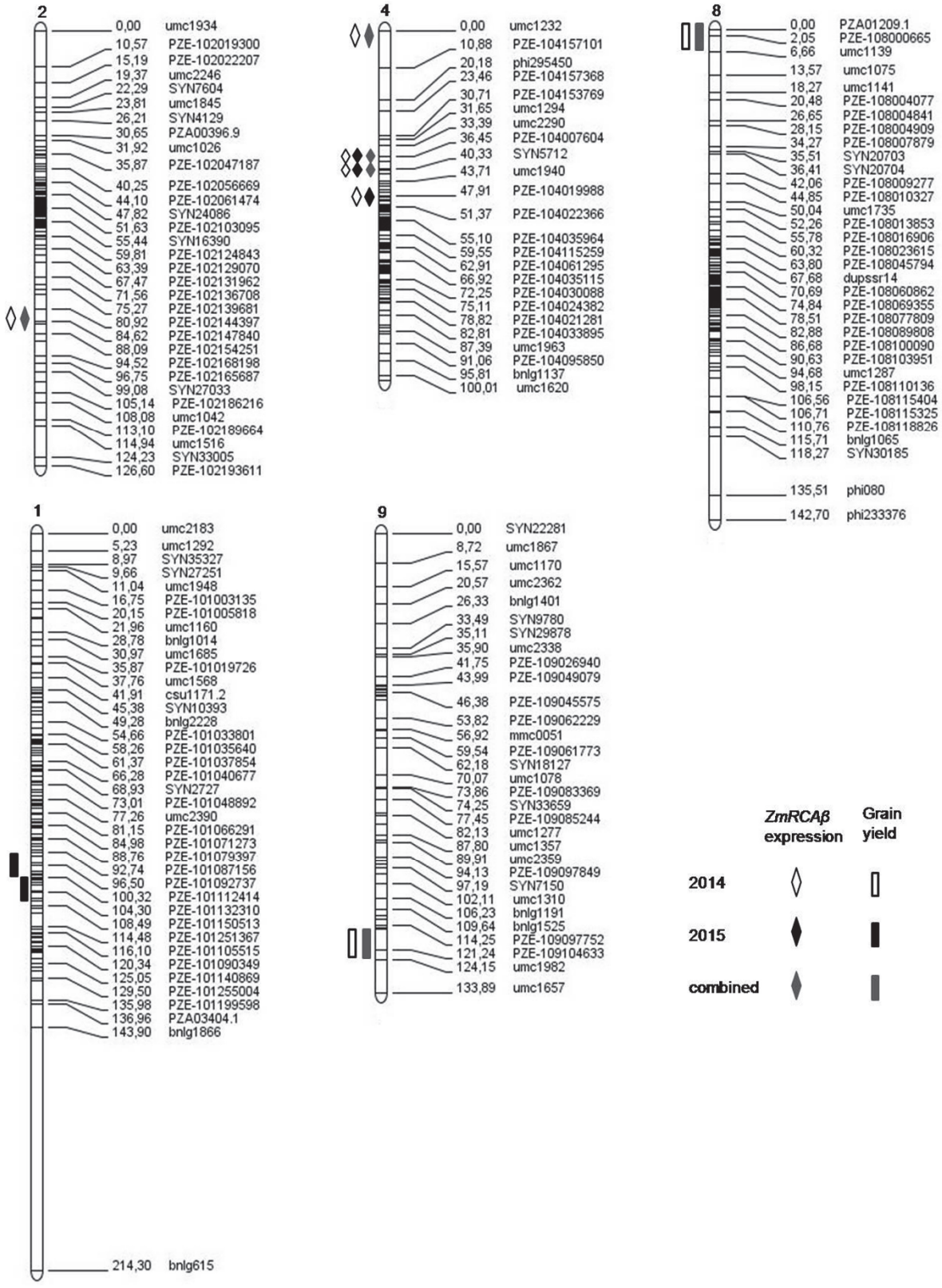

Fig. 1. Summary of eQTL/QTL locations detected. Chromosome number is shown above each chromosome. Genetic distance and markers are shown to the right of each chromosome (not all markers are displayed). eQTLs/QTLs, represented by bars or diamonds, are illustrated to the left of the chromosome. The eQTLs/QTLs detected in 2014, 2015, and in combined environments are represented by white, black, and gray, respectively. Chromosomes with no eQTLs/QTLs are omitted from this figure.

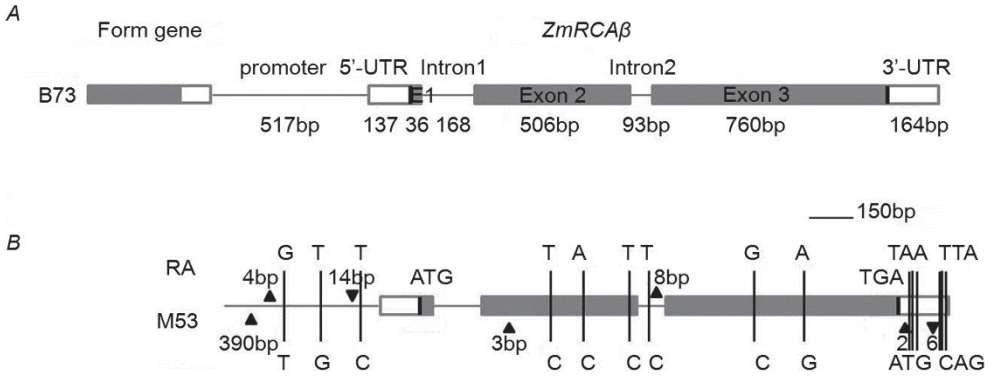

Fig. 2. Gene structure and polymorphisms of *ZmRCAβ* in RA and M53. The gray and open boxes indicate the exons and the UTRs, respectively. *A*. Gene structure predicted from the released genome sequence of the inbred line B73 (http://www.phytozome.net/). The promoter, 5´-UTR, exon1, intron1, exon 2, intron 2, exon 3, and 3´-UTR of *ZmRCAβ* are 517 bp, 137 bp, 36 bp, 168 bp, 509 bp, 93 bp, 760 bp, and 164 bp, respectively. *B*. Polymorphic sites in the gDNA of *ZmRCAβ* between RA and M53. The vertical lines represent SNPs. The triangles represent indels. The upper triangles indicate deletions from RA relative to M53, and the lower triangles indicate deletion from M53 relative to RA. The numerals indicate the number of deleted nucleotides.

programs. The identified eQTLs for *ZmRCAβ*, especially those repeatedly detected in different environments, make possible to improve RCA expression by marker-assisted breeding methods such as QTL pyramiding, which is a process of assembling several QTLs for a specific trait from different loci to produce superior genotypes (Xu *et al.* 1997). However, our study constitutes only first-order knowledge on the genetic determinism of *ZmRCAβ* expression levels in maize. Considering that the eQTL/QTLs detected in different materials and in multiple environments are the most valuable for breeding, further eQTL mapping of *ZmRCAβ* in a range of maize materials under different environments is warranted.

Phenotypic differences between individuals are not only due to sequence polymorphisms that produce altered (or absent) proteins but also gene expression difference that generates varying amounts of protein in a cell or tissue (Druka *et al.* 2010). Thus, regulating transcript abundance is an effective approach to improve phenotypes. In the past decade, many important genes associated with important traits have been cloned, such as disease resistance (Zuo *et al*. 2015), quality (Wang *et al*. 2014), and grain yield (Li *et al*. 2010). eQTL mapping can provide an approach to improve phenotypes *via* the modulation of the expression of these important genes. In the present study, we mapped the eQTLs for maize RCA genes in different field growing seasons and detected stably expressed eQTLs. The results provide new ideas for helping to regulate the expression of important genes through molecular markers in breeding practices.

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